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The presence of Cronobacter *sakazakii*, Enterobacteriaceae spp. and Ochratoxin-A in Infant Rice-based formula and milled rice products

Sibel Özçakmak[1] and Asya Çetinkaya[2]
[1]Provincial Directorate of Agriculture and Livestock, Samsun, Türkiye
[2]Kafkas University, Department of Food Engineering, Kars, Turkey

ABSTRACT

Rice based products could be have microbiological and toxicological hazards causing for unsuitable production and storage conditions. In this study, samples of three different commercial Infant Rice-based formulae with four samples (IRF), nine milled rice (MR) samples were searched for Cronobacter sakazakii, Enterobacteriaceae spp. and Ochratoxin-A (OTA). The presence of C. sakazakii and Enterobacteriaceae spp. was detected using ISO/TS 22964/IDF/RM 210 and and ISO 21528-2: 2004 method, respectively. A developed method using OCHRAPREP® (REFN: A9-P14. V4) in conjunction with HPLC described by AOAC 2000.003 was used with slight modifications of the purification and HPLC conditions and with another confirmation method for OTA. The limit of quantities measurement was 1.286 µg/kg. C. sakazakii was not found in any samples. Enterobacteriaceae spp. was determined above the legal limits (between 2.6x102 and 2.8x104 kob/g, average 3.46x104) in four samples (30.77%). OTA was detected by one IRF with milk product sample with 0.60 µg/kg (7.69%).

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***Correspondence to**: Sibel Özçakmak, Provincial Directorate of Agriculture and Livestock, Samsun, Türkiye*
Tel: +90 (362) 231-3700
Fax: +90 (362) 231-3700
E-Mail: damla-damla49@hotmail.com

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INTRODUCTION

The significance and potential health risk of any contaminants in baby foods has been reported in the literature [1, 2]. A number of foodborne outbreaks associated with cereals based infant formula have been increased. Some mycotoxins [such as deoxynivalenol, zearalenone, ochratoxin A [OTA] and Cronobacter sakazakii have been reported to be prevalent at verry low concentrations in infant cereal foods [3, 4, 5, 6, 7] . OTA has been reported to be prevalent at very low concentrations in infant cereal foods [8].

Mycotoxins are naturally occurring chemicals produced by moulds growing on foodstuffs, including rice. Among these rice mycotoxins, OTA is classified by the International Agency for Research on Cancer as a possible human carcinogen (Category 2B). OTA has been shown to have nephrotoxic, immunotoxic, genotoxic and teratogenic properties [9]. OTA was present semolina based baby foods, multicereal formulas, rice formulas [10]. If milled rice used for the production of infant formulas is subject to OTA contamination cause of fungi contamination, the same is to be expected for the infant formula [11]. In particular, there is scarce information on the presence of OTA in rice based baby foods and/or ground rice products.

Enterobacter sakazakii is a member of the family Enterobacteriaceae, genus Enterobacter, and is a motile peritrichous, gram-negative bacillus [12]. Cronobacter sakazakii, formerly E. sakazakii [13] has been isolated commonly in contaminated powdered infant formula [6]. C. sakazakii was isolated from rice seed [dried product] [5], infant food [14], rice, rice starch and rice flour [6, 12]. C. sakazakii is considered an opportunistic pathogen that has been implicated in severe forms of necrotizing colitis and meningitis, especially in neonates [1].

Outbreaks of infections have implicated powdered milk substitute infant formulas as vehicles of C. *sakazakii* [14]. Many countries regulate specific mycotoxins and most countries try to limit exposure to the toxins. Community maximum levels for certain contaminants

in foodstuffs were established by Commission Regulation [EC] No 1881/2006 of 19 December 2006 [15]. Turkish Food Codex [TFC] Regulation on Contaminants [16] was prepared in parallel with the European Union Commission Regulation 1881/2006/EC. TFC Regulation on Contaminants in foods (Law of Authorization: 5996 and Official Gazette of Publication: 29.12.2011-28157) sets a limit of 5μg/g for OTA in unprocessed cereals, 3 μg/g for products (except those listed below) and 0.5 μg/g for food supplements for infants and young children and dietary foodstuffs for special medical purposes for infants.

TFC Regulation on Microbiological Criteria was parallel with the EC Regulation 2073/2005/EC on Microbiological Criteria for Foodstuffs. Accordingly, *C. sakazakii* as a Food Safety Criteria mustn't be in infant formulae and follow-up formulae. Enterobacteriaceae spp. for food supplements for infants and children [including dietary foodstuffs for special medical purposes] mustn't exceed 10^1cfu g^{-1}.

The objective of this study was to investigate the prevalence of *C. sakazakii*, Enterobacteriaceae spp. and OTA in commercial Infant Rice-based formula (IRF) and milled rice (MR) products. A total of 10 samples were obtained from market, two samples were get flour mill manufacture area and one from storage/warehouse place.

MATERIALS AND METHODS

All of the analyses were performed using accreditation methods. Accreditation Number given by Turkish Accreditation Agency (TURKAK) is AB-0566-T and Revision Number is 07. All media materials used in the study were obtained from Oxoid, UK.

Sample collection

In this study, a total of 4 different random commercial Infant Rice-based formula and 6 milled rice packaged products were purchased from retail stores. Two of milled rice products were provided by a mill manufacture area and one of storage/sales place. The samples between 125 and 250 g weights collected in November and December 2016 were manufactured in 4 different cities. Sample information is given in Table 1. Collected samples were taken to the laboratory without delay in accordance with Regulation on Turkish Food Codex Microbiological Criteria [17]. Each sample was labeled to identify the source, site and date of sampling. The other information were added Appendix 1.

Detection, isolation and identification of *C. sakazakii*

The presence of *C. sakazakii* was detected using ISO/TS 22964/IDF/RM 210 method [18] (Milk and milk products-Detection of E. *sakazakii*). Typical colonies on a chromogenic isolation agar, form yellow colonies on tryptone soya agar and display biochemical characteristics as described, when tests are carried out in accordance with this technical specification. In accordance with the interpretation of the test results of isolation of presumptive *C. sakazakii*, report the presence or absence of presumptive *C. sakazakii* in the test portion. In this case, no confirmation of the presumptive *C. sakazakii* found on the chromogenic plate has been carried out. After

Table 1. Information about investigated samples.

Sample_id	*Sample*	*Country*	*Year*	*Brand*	*City*
1	*Fresh Milled rice flour*	*Turkey*	*2016*	*Medium scale*	*Samsun*
2	*Fresh Milled rice flour*	*Turkey*	*2016*	*Medium scale*	*Samsun*
3	*Milled rice flour in bag*	*Turkey*	*2016*	*Medium Scale*	*Samsun*
4	*Rice flour with milk and banana*	*Turkey*	*2016*	*Big scale*	*Ankara*
5	*Rice flour with milk, 12 vitamins and 6 minerals*	*Turkey*	*2016*	*Big scale*	*İstanbul*
6	*Supplementary food with milk and rice for baby and small children*	*Turkey*	*2016*	*Big scale*	*Ankara*
7	*Rice flour with milk and 7 cereals (for night)*	*Turkey*	*2016*	*Big scale*	*İstanbul*
8	*Rice flour*	*Turkey*	*2016*	*Big scale*	*İstanbul*
9	*Rice flour*	*Turkey*	*2016*	*Big scale*	*İstanbul*
10	*Rice flour*	*Turkey*	*2016*	*Big scale*	*Ankara*
11	*Rice flour*	*Turkey*	*2016*	*Big scale*	*Kocaeli*
12	*Rice flour*	*Turkey*	*2016*	*Big scale*	*İstanbul*
13	*Rice flour*	*Turkey*	*2016*	*Big scale*	*İstanbul*

confirmation test, of one or more of the presumptive C. *sakazakii*, report the presence or absence of C. *sakazakii* in the test portion. Specify the final test result per mass [in grams] or per volume [in millilitres] of the analysed test sample.

Detection and Enumeration of *Enterobacteriaceae* spp.

Microorganisms that form characteristic colonies on violet red bile glucose agar and that ferment glucose and show a negative oxidase reaction when the tests are carried out in accordance with the methods specified in ISO 21528-2:2004 [19]. Number of Enterobacteriaceae counts in per gram of the test sample is calculated from the number of confirmed typical colonies per dish.

Determine OTA levels

The aim of the following work was to determine OTA levels in the samples from manufacture, sales and storage place in Turkey. A developed method using OCHRAPREP® (REFN: A9-P14.V4) in conjunction with HPLC described by AOAC 2000.003 [20] was used with slight modifications of the purification and HPLC conditions and with another confirmation method for OTA. The limit of quantitive measurement (LOQ) was 1.286 µg/kg. The method has been assessed and found to be suitable for the detection of OTA in cereal, offering average recoveries of 88%. The actual concentration of ochratoxin A is calculated using the following equation.

Calculate the concentration of the ochratoxin A standard using the following calculation:

$$[Ochratoxin\ A](\frac{\mu g}{ml}) = \frac{Abs(333\,\text{nm}) \times \text{Molecular Weight} \times 1000}{Ochratoxin\ A\ Extinction\ coefficient}$$

$$= \frac{Abs(333\,\text{nm}) \times 403 \times 1000}{5550}$$

$$= Abs(333\,\text{nm}) \times 72.6$$

RESULTS

Table 2 summarizes the result of C. *sakazakii*, Enterobacteriaceae spp. and OTA values in Infant rise-based formula (IRF) and milled rice (MR) samples. C. *sakazakii* was not determined in any IRF and MC products. While C. sakazakii wasn't detected from any samples, *Enterobacteriaceae* spp. was determined average 3.46x10^4 cfu/g in four samples (30.77%) that was above the legal limit value (max 10^1 cfu/g).

C. *sakazakii* was not isolated from any samples (n=13).

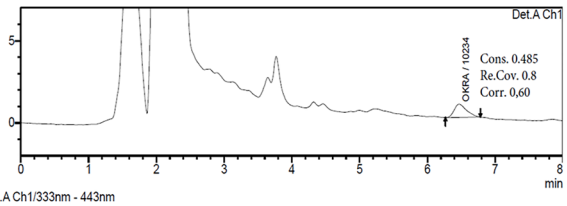

Figure 1. Chromatogram obtained from MR samples of 6 spiked with OTA.

The negative strains of C. *sakazakii* formed yellow colonies on TSA after 24–72 h of incubation at 25°C and blue-green colonies after 24 h of incubation at 37°C on DFI medium. The determination of high level of Enterobacteriaceae in IRF and MR has potential hazardous for pathogen foodborne enteric bacteria especially for C. *sakazakii*. The number of Enterobacteriaceae spp. was average 3.46x10^4 cfu/g. 4 of 13 samples (30.77%) was exceed EC limits (10^1 cfu/g). One of the samples (7.69%) taken from market had levels of mycotoxin (0.6 µg/kg) above maximum level for certain contaminants in foodstuffs (0.5 µg/kg) (Figure 1). OTA contamination in the other samples wasn't present.

DISCUSSION

A great deal of studies has focused on the infant formula as the main source of serious pathogens [3, 5, 11]. But, few researchers a little researcher focused on the infant formula including ground cereal such as rice, semolina etc. for the main source of pathogen [14, 19, 21]. Rice flour is commonly used as an infant formulae ingredient. If it is contaminated in any production stage, the microbiological hazard and risk could be transported to the last product. Nazarowec-White and Farber [22] stated that *E. sakazakii* can gain access to the powder from the environment or from the addition of the ingredients at the powder stage. Iversen and Forsythe [10] reported that the presence of *E. sakazakii* in powdered infant milk formula depends on the process conditions and nature of the product. The bacterium has been isolated from rice [5] but its survival characteristics in infant rice cereal are not known. Post processing contamination of rice cereal with *C. sakazakii*, followed by survival throughout the expected shelf-life of the product, would result in its presence.

The presence of *Enterobacteriaceae* spp. respects the poor hygienic conditions of the environment of factories and cross contamination [7]. The manufacture environments taken by MR samples and also personnel hygienic conditions weren't sufficient. The mill equipment used in rice flour was not suitable for good manufacturing practices. The contamination by *Enterobacteriaceae* means that it should be designated sampling plans and limits for the level of *Enterobacteriaceae* spp. in baby foods for food business operators as part of the process hygiene criteria. The initial *Enterobacteriaceae* contamination level in the raw materi-

Table 2. The results for *C. sakazakii*, *Enterobacteriaceae* spp. and OTA analysis

Sample id	Analyte	is less than (legal limit)	Determinated Value	Units	Uncertainty
	C. sakazakii	0	not detected	g mL^{-1}	
1	Enterobacteriaceae spp.	100	$5.6x10^{8}$	cfu g^{-1}	
	Ochratoxin-A	0.5	not detected	µg kg^{-1}	±0.156%
	C. sakazakii	0	not detected	g mL^{-1}	
2	Enterobacteriaceae spp.	100	$7.5x10^{2}$	cfu g^{-1}	
	Ochratoxin-A	0.5	not detected	µg kg^{-1}	±0.156%
	C. sakazakii	0	not detected	g mL^{-1}	
3	Enterobacteriaceae spp.	100	<10	cfu g^{-1}	
	Ochratoxin-A	0.5	not detected	µg kg^{-1}	±0.156%
	C. sakazakii	0	not detected	g mL^{-1}	
4	Enterobacteriaceae spp.	100	<10	cfu g^{-1}	
	Ochratoxin-A	0.5	not detected	µg kg^{-1}	±0.156%
	C. sakazakii	0	not detected	g mL^{-1}	
5	Enterobacteriaceae spp.	100	$2.6x10^{2}$	cfu g^{-1}	
	Ochratoxin-A	0.5	not detected	µg kg^{-1}	±0.156%
	C. sakazakii	0	not detected	g mL^{-1}	
6	Enterobacteriaceae spp.	100	<10	cfu g^{-1}	
	Ochratoxin-A	0.5	0,6	µg kg^{-1}	±0.156%
	C. sakazakii	0	not detected	g mL^{-1}	
7	Enterobacteriaceae spp.	100	$2.8x10^{4}$	cfu g^{-1}	
	Ochratoxin-A	0.5	not detected	µg kg^{-1}	±0.156%
	C. sakazakii	0	not detected	g mL^{-1}	
8	Enterobacteriaceae spp.	100	<10	cfu g^{-1}	
	Ochratoxin-A	0.5	not detected	µg kg^{-1}	±0.156%
	C. sakazakii	0	not detected	g mL^{-1}	
9	Enterobacteriaceae spp.	100	<10	cfu g^{-1}	
	Ochratoxin-A	0.5	not detected	µg kg^{-1}	±0.156%
	C. sakazakii	0	not detected	g mL^{-1}	
10	Enterobacteriaceae spp.	100	<10	cfu g^{-1}	
	Ochratoxin-A	0.5	not detected	µg kg^{-1}	±0.258%
	C. sakazakii	0	not detected	g mL^{-1}	
11	Enterobacteriaceae spp.	100	<10	cfu g^{-1}	
	Ochratoxin-A	0.5	not detected	µg kg^{-1}	±0.258%
	C. sakazakii	0	not detected	g mL^{-1}	
12	Enterobacteriaceae spp.	100	<10	cfu g^{-1}	
	Ochratoxin-A	0.5	not detected	µg kg^{-1}	±0.258%
	C. sakazakii	0	not detected	g mL^{-1}	
13	Enterobacteriaceae spp.	100	<10	cfu g^{-1}	
	Ochratoxin-A	0.5	not detected	µg kg^{-1}	±0.258%

als is predominantly governed by Good Hygiene Practices [23].

Cereal based infant products are potantial sources for mycotoxins such as OTA [3, 24]. Although some researchers have found the contamination of OTA and the other mycotoxins in retailed rice [25, 26], a smaller survey of the actual incidence of mycotoxins in IRF has been reported. In a survey by Lombaert et al. [3] demonstrated the regular occurrence of multiple mycotoxins in cereal-based infant foods. In recent publications, OTA has been reported to be prevalent at very low concentrations in infant and baby cereal foods. Wolff [7] and Beretta et al. [8] have been established the maximum OTA levels as 2.13 and 0.7 ng/g, respectively. The OTA concentration in IRF sample found in this investigation (0.6 ng/g) was higher than these results. Rice-based infant formulae contained higher amount than legal limit of OTA as determined in this study. Although the production companies of the samples had a HACCP management system certification, microbiological hazard criteria was not acceptable according the TFC limits.

CONCLUSION

Microbiological risk in baby foods especially with grain mixed should be widely searched to exhibit potential risks. It should be followed up all of the processing steps in order to prevent contamination to the last product. The findings of this study suggest that the flour mill conditions have poor hygiene. The initial *Enterobacteriaceae* contamination level in the raw materials is predominantly governed by Good Hygiene Practices. Mycotoxin contamination should be more following up by food business operators. *C. sakazakii*, *Enteobacteriaceae* spp. and OTA analyses in infant foods must be involved in provincial annual control plan by Republic of Turkey Ministry of Food, Agriculture and Livestock.

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